Tau peptides and tau mutant protein aggregation inhibition by cationic polyethyleneimine and polyarginine

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Abstract

Tau protein plays a major role in Alzheimer's disease. The tau protein loses its functionality by self-aggregation due to the two six-amino acid sequences VQIVYK, and VQIINK of the protein. Hence its imperative to find therapeutics that could inhibit the self-aggregation of this tau peptide fragments. Here we study the inhibitory potential of a cationic polymer polyethyleneimine (PEI), and a cationic polypeptide arginine (Arg) on the aggregation of VQIVYK, and GKVQIINKLDL peptides, and tau mutant protein (P301L), found frequently in taupathy. Various characterization methods are employed including thioflavin T, transmission electron microscopy (TEM), and dynamic light scattering (DLS) to study the aggregation/inhibition process *in vitro*. Results show, PEI and Arg significantly inhibit tau peptides and protein aggregation. The study could be applied to understand tau protein aggregation mechanism in the presence of cationic polymers.

Key words: Aggregation, tau peptide, polyethyleneimine, polyarginine, inhibition, cation

Introduction

Tau protein plays an important role in the regulation of microtubules in the brain. During Alzheimer's and related pathological conditions, tau has been shown to dissociate from the microtubule, self-aggregates, and form neurofibrillary tangles [1, 2]. Cell to cell transmission of the aggregates and fibrillar structures results in neuronal cell death and subsequent progression of the Alzheimer's [3, 4]. The hexapeptide repeats VQIVYK, and VQIINK found in the carboxyl region of the tau protein, believe to be the drivers in the formation of inter-tau beta sheets, and subsequent aggregation and fibril structures [2, 5, 6]. The hexapeptide repeats are well characterized, and have shown to form beta-sheets, and subsequent fibrillar assembly with cross beta sheet structure, *in vitro* [6, 7]. They are widely used as a model to study tau aggregation, and for the investigation of therapeutic interventions of this pathologic aggregation [4, 6, 8].

Several therapeutic modalities for the inhibition of tau peptide/protein aggregation have been reported [9, 10]. Among the approaches, the use of small molecule compounds has recently gained a lot of interest. Several small molecules have shown to exhibit anti-tau aggregation properties against tau peptides and protein [11, 12]. ~~Although small molecules have been shown to have tau aggregation inhibitory effects, the use of high dose, and reduced half-life, often results in toxicity and side effects~~. However, there have been only few studies reported on the effect of macromolecules on tau aggregation mechanism and in tau pathology [13-15]. Recent study by Dammers and others show the

modulation of tau aggregation mechanism by D-enantiomer peptides [13]. Other macromolecules that have been shown to have effect on tau pathology are, an eight amino acid peptide Davunetide [14], and a D-aminoacid inhibitor peptide [15]. By studying the macromolecules-tau interaction, further insights on tau aggregation mechanism and inhibition could be unraveled, and could aid in the development of tau aggregation inhibitory molecules, and drug delivery strategies. Previous studies have shown that cationic small molecules, and cationic osmolyte urea have inhibitory potential on tau aggregation [8, 16, 17]. However effect of cationic polymers on tau peptide aggregation inhibition hasn't been explored in detail yet. We are particularly interested in studying the cationic polyethyleneimine and polyarginine polymers on their effect on tau aggregation.

Here in this paper we investigated the inhibitory potential of the cationic polymers PEI and PR on the aggregation of tau hexapeptide domain ^{306}VQIVYK^{311} (Tau V) segment from the microtubule binding region of tau protein, and the peptide fragment GKVQIINKLDL (Tau N), which is widely found in taupathy. The peptides are well characterized and have been used as models to study the tau aggregation [6, 8]. In addition, to have a physiologically closer mimic, we also investigate the aggregation inhibition of a mutant tau protein (P301L), found in adult brains affected by taupathy [18, 19]. The aggregation with and without the cationic polymer, and polypeptide/protein were studied utilizing the standard peptide aggregation characterization methods thioflavin S, TEM, and dynamic light scattering (DLS). The results show that the cationic PEI and Arg, exhibited significant inhibitory effect on the tau peptides and protein

aggregation. The study demonstrates for the first time that cationic polymers could inhibit tau peptide and protein aggregation, and could be used as model compounds to study the tau aggregation mechanism *in vitro*.

Materials and Methods

Materials

Tau peptides, and corresponding mutant peptides were custom synthesized from Genscript. Tau mutant protein, P301L was obtained from rPeptide. Low molecular weight heparin was purchased from Galen laboratory supplies. All other chemicals, and reagents were purchased from Sigma Aldrich. The polyethyleneimine-branched (Mw 25,000, cat no: 408727), and poly-l-arginine hydrochloride (Mw 5,000-15,000, cat no: P4663) were obtained from Sigma Aldrich, and was used as obtained.

Thioflavin S (ThS) fluorescence measurements

Tau peptides stocks of 1 mM were prepared by dissolving in deionized water. ThS of 0.5 mg/ml was freshly prepared with 20 mM MOPS buffer, pH 7. 1 mM Heparin stock solutions were prepared by dissolving in deionized water. For the ThS assay, 10 µl of peptide was dissolved in 2.5 µl heparin, 10 µl ThS, and MOPS buffer with or without the cationic inhibitors to make the final 100 µl working solution. ThS fluorescence was measured at 440/490 nm excitation and emission using a spectrophotometer in the lab. Kinetic measurements were performed with 2 minutes interval for 30 min. The experiments were repeated for three independent experiments.

DLS

DLS measurements were performed using a Malvern zetasizer instrument in the lab. Samples were prepared under similar conditions without the ThS, and diluted 10 times, and the size measurements were performed. The experiments were repeated for three independent experiments.

TEM

For TEM imaging, the peptides, after 2 hours of aggregation were spotted on holy carbon copper grids, and then stained with 1% uranyl formate. The samples were then be imaged using the JEOL JSM 1400 TEM at UM-Ann arbor electron microscope facility.

Cell toxicity assay

The toxicity of PEI and Arg on normal human neuroblastoma SH-SY5Y cells was studied by the XTT assay. Cells were obtained from the American Type Culture Collection (ATCC), and were cultured according to the manufacturers protocol. We have also tested the toxicity effect of Tau N on these cells. For the study, $2x10^4$ cells/well, were cultured in 96 well plates over night. For PEI and Arg toxicity, PEI concentrations of 50-200 nM, and Arg concentrations of 1- 4 μM were incubated for 48 hours. For Tau N toxicity study, Tau N concentrations of 10, 20, and 40 μM were tested with and without PEI and Arg. XTT assay was performed at 470 nm, and the viability was determined according to manufacture's protocol.

Statistical Analysis

Data were collected from three or more replicates for each experiment, and they are presented as mean ± standard error of the mean (SEM). P-values were determined from the results of at least 3-independent experiments for statistical significance unpaired T-test was used.

Results and Discussion

The approach used for the study is depicted in Figure 1. The goal of the study is to test the potential of cationic polymer PEI and cationic peptide Arg on the inhibition of tau peptide/protein aggregation, and subsequent fibril formation. First, we performed thioflavin S measurements to probe the aggregation kinetics of the tau peptides with and without PEI and Arg. The thioflavin S measurements show that the cationic polymers successfully inhibit both Tau V, and Tau N peptides aggregation *in vitro*. The kinetic study show, faster aggregation for peptides without any inhibitors. PEI with hundred times lower molar concentrations was able to inhibit both peptides aggregation, while Arg with about ten times lower molar concentrations was able to inhibit the peptides Tau N (Figure 2), and Tau V (Figure 3) aggregation. To confirm, that the aggregation of Tau N, and Tau V is sequence specific, we did scrambled peptides VKYVIQ, and GKIVQNIVLKKLD aggregation. As can be seen from Figure 4, the scrambled peptides did not show significant aggregation, indicating the importance of hexapeptides domain in tau aggregation.

We then tested the morphology of the Tau N and Tau V aggregates with and without PEI and Arg using TEM. Images reveal, that the tau peptide Tau N fibers formed more fibrils

compared to the Tau V aggregates, corroborating the ThS data. PEI, and Arg in few micromolar concentrations were able to effectively inhibit the aggregation of 100 μM peptides as seen in Figure 5, and is in agreement with the ThS data. Further, as a complementary technique, we performed dynamic light scattering measurements to study the size of the peptides aggregation with and without PEI and Arg. The size measurements for GKVQIINKLDL (Tau N) aggregation show, that the aggregation size is reduced in the presence of polyethyleneimine or polyarginine, in corroboration with the ThS and TEM measurements (Figure 6).

We then studied the inhibitory effect of the cationic polymers on tau mutant protein aggregation. The aggregation inhibition of a mutant tau protein (P301L), found in adult brains affected by taupathy was studied. To test the effect of aggregation inhibition, we performed aggregation kinetics of tau mutant protein aggregation with and without PEI and Arg similar to the peptide aggregation experiments described earlier. As can be seen from ThS measurements (Figure 7A), DLS (Figure 7B), and TEM images (Figure 7C), the cationic polymers are able to inhibit the protein aggregation.

Finally, in order to assess whether the cationic peptides have toxicity effects in normal cells, we performed cellular toxicity studies on normal cells in the presence of PEI and Arg with varying concentrations. For the study, we used SH-SY5Y human neuroblastoma cells, which is also used as a model cell line in neurodegenerative disease studies [20], and hence would be a suitable for our study. Cells were cultured in 96 well plates and incubated with PEI and Arg for 48 hours and XTT assay was performed to assess the cell

viability. As can be seen from the Figure 8, no significant toxicity was observed from PEI or Arg up to a few micro molar concentrations. We have also tested the toxicity effects of Tau N on SH-SY5Y cells (Figure 9). Tau N exhibited toxicity around 40 μM of peptide concentration, while the lower concentrations showed no toxicity (Figure 9A). Tau N treated with PEI and Arg did not exhibit cellular toxicity at the tested concentrations (Figure 9B, C). Hence, the cationic polymers, could have therapeutic potential against tau aggregation.

Here we show that cationic polymers have the capability to inhibit tau mutant fibrillization *in vitro*. It is widely known that microtubules and polyanions contribute to the neurofibrillary tangle formation [21, 22], and glycosaminoglycans, which are anionic in nature, believed to play a key role in the tau aggregation [23, 24]. Hence, by interacting with the glycosaminooglycan heparin that is used in the study, the cationic PEI and Arg could have prevented the tau peptides and protein aggregation. Previously, it has been reported that several cationic small molecule compounds such as, cyanine, phenothiazine, and arylmethine in micromolar concentrations, exhibited inhibitory effects by interacting with the nucleation process mediated by the tau peptides [16]. Another recent study report the inhibitory effect of urea, a cationic osmolyte on tau fibrillization [8]. These studies further indicate the influence of cationic molecules on tau aggregation inhibition, and the current study would provide new insights and future investigation on the influence of cationic macromolecules on tau aggregation. ~~The cationic polymers may have hydrophobic domains that could act as binding pockets for the aggregation prone peptide, hence preventing nucleation, and subsequent aggregation kinetics.~~

Conclusion

The cationic polymers PEI and Arg, exhibited inhibitory effects on tau peptides, and protein aggregation *in vitro*, and could aid in understanding the mechanism of tau aggregation inhibitory process. Further, they could to be used as drug carriers for treating tau aggregation. PEI and Arg have shown potential in gene delivery [25-28], and hence could be used in Alzheimer's gene therapy. Arg is also known for its cell penetrating capabilities [29, 30], and could be used as a drug depot for effective intracellular delivery of therapies for tau aggregation, which is mainly observed inside the cells [4, 19]. From this proof of concept study, we show that the cationic polymeric compounds may serve as potential inhibitors for tau peptide aggregation, and could be applied to study the mechanism of tau protein aggregation.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgement

We would like to thank the University of Michigan Dearborn department of Mechanical engineering, and the College of Engineering and Computer Science for funding. We would like to thank the University of Michigan Microscope and Imaging (MIL) facility for the use of TEM microscope.

References:

1. Kolarova, M., Garcia-Sierra, F., Bartos, A., Ricny, J. & Ripova, D. (2012) Structure and pathology of tau protein in Alzheimer disease, *Int J Alzheimers Dis.* **2012**, 731526.
2. von Bergen, M., Barghorn, S., Biernat, J., Mandelkow, E. M. & Mandelkow, E. (2005) Tau aggregation is driven by a transition from random coil to beta sheet structure, *Biochim Biophys Acta.* **1739**, 158-166.
3. Pooler, A. M., Polydoro, M., Wegmann, S., Nicholls, S. B., Spires-Jones, T. L. & Hyman, B. T. (2013) Propagation of tau pathology in Alzheimer's disease: identification of novel therapeutic targets, *Alzheimers Res Ther.* **5**, 49.
4. Mohamed, N. V., Herrou, T., Plouffe, V., Piperno, N. & Leclerc, N. (2013) Spreading of tau pathology in Alzheimer's disease by cell-to-cell transmission, *Eur J Neurosci.* **37**, 1939-1948.
5. Mandelkow, E., von Bergen, M., Biernat, J. & Mandelkow, E. M. (2007) Structural principles of tau and the paired helical filaments of Alzheimer's disease, *Brain Pathol.* **17**, 83-90.
6. Li, W. & Lee, V. M. (2006) Characterization of two VQIXXK motifs for tau fibrillization in vitro, *Biochemistry.* **45**, 15692-156701.
7. Goux, W. J., Kopplin, L., Nguyen, A. D., Leak, K., Rutkofsky, M., Shanmuganandam, V. D., Sharma, D., Inouye, H. & Kirschner, D. A. (2004) The formation of straight and twisted filaments from short tau peptides, *J Biol Chem.* **279**, 26868-26875.
8. Eschmann, N. A., Do, T. D., LaPointe, N. E., Shea, J. E., Feinstein, S. C., Bowers, M. T. & Han, S. (2015) Tau Aggregation Propensity Engrained in Its Solution State, *J Phys Chem B.* **119**, 14421-14432.
9. Boutajangout, A. & Wisniewski, T. (2014) Tau-based therapeutic approaches for Alzheimer's disease - a mini-review, *Gerontology.* **60**, 381-385.
10. Brunden, K. R., Ballatore, C., Crowe, A., Smith, A. B., 3rd, Lee, V. M. & Trojanowski, J. Q. (2010) Tau-directed drug discovery for Alzheimer's disease and related tauopathies: a focus on tau assembly inhibitors, *Exp Neurol.* **223**, 304-310.
11. Mohamed, T., Hoang, T., Jelokhani-Niaraki, M. & Rao, P. P. (2013) Tau-derived-hexapeptide 306VQIVYK311 aggregation inhibitors: nitrocatechol moiety as a pharmacophore in drug design, *ACS Chem Neurosci.* **4**, 1559-1570.
12. Mohamed, T. & Rao, P. P. (2011) Alzheimer's disease: emerging trends in small molecule therapies, *Curr Med Chem.* **18**, 4299-4320.
13. Dammers, C., Yolcu, D., Kukuk, L., Willbold, D., Pickhardt, M., Mandelkow, E., Horn, A. H., Sticht, H., Malhis, M. N., Will, N., Schuster, J. & Funke, S. A. (2016) Selection and Characterization of Tau Binding -Enantiomeric Peptides with Potential for Therapy of Alzheimer Disease, *PLoS One.* **11**, e0167432.
14. Matsuoka, Y., Jouroukhin, Y., Gray, A. J., Ma, L., Hirata-Fukae, C., Li, H. F., Feng, L., Lecanu, L., Walker, B. R., Planel, E., Arancio, O., Gozes, I. & Aisen, P. S. (2008) A neuronal microtubule-interacting agent, NAPVSIPQ, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer's disease, *J Pharmacol Exp Ther.* **325**, 146-153.
15. Sievers, S. A., Karanicolas, J., Chang, H. W., Zhao, A., Jiang, L., Zirafi, O., Stevens, J. T., Munch, J., Baker, D. & Eisenberg, D. (2011) Structure-based design of non-natural amino-acid inhibitors of amyloid fibril formation, *Nature.* **475**, 96-100.

16. Schafer, K. N., Cisek, K., Huseby, C. J., Chang, E. & Kuret, J. (2013) Structural determinants of Tau aggregation inhibitor potency, *J Biol Chem.* **288**, 32599-32611.
17. Levine, Z. A., Larini, L., LaPointe, N. E., Feinstein, S. C. & Shea, J. E. (2015) Regulation and aggregation of intrinsically disordered peptides, *Proc Natl Acad Sci U S A.* **112**, 2758-2763.
18. Lewis, J., McGowan, E., Rockwood, J., Melrose, H., Nacharaju, P., Van Slegtenhorst, M., Gwinn-Hardy, K., Paul Murphy, M., Baker, M., Yu, X., Duff, K., Hardy, J., Corral, A., Lin, W. L., Yen, S. H., Dickson, D. W., Davies, P. & Hutton, M. (2000) Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein, *Nat Genet.* **25**, 402-405.
19. Mirra, S. S., Murrell, J. R., Gearing, M., Spillantini, M. G., Goedert, M., Crowther, R. A., Levey, A. I., Jones, R., Green, J., Shoffner, J. M., Wainer, B. H., Schmidt, M. L., Trojanowski, J. Q. & Ghetti, B. (1999) Tau pathology in a family with dementia and a P301L mutation in tau, *J Neuropathol Exp Neurol.* **58**, 335-345.
20. Schlachetzki, J. C., Saliba, S. W. & Oliveira, A. C. (2013) Studying neurodegenerative diseases in culture models, *Rev Bras Psiquiatr.* **35 Suppl 2**, S92-100.
21. Mukrasch, M. D., Biernat, J., von Bergen, M., Griesinger, C., Mandelkow, E. & Zweckstetter, M. (2005) Sites of tau important for aggregation populate {beta}-structure and bind to microtubules and polyanions, *J Biol Chem.* **280**, 24978-24986.
22. Barghorn, S. & Mandelkow, E. (2002) Toward a unified scheme for the aggregation of tau into Alzheimer paired helical filaments, *Biochemistry.* **41**, 14885-14896.
23. Paudel, H. K. & Li, W. (1999) Heparin-induced conformational change in microtubule-associated protein Tau as detected by chemical cross-linking and phosphopeptide mapping, *J Biol Chem.* **274**, 8029-8038.
24. Papy-Garcia, D., Christophe, M., Huynh, M. B., Fernando, S., Ludmilla, S., Sepulveda-Diaz, J. E. & Raisman-Vozari, R. (2011) Glycosaminoglycans, protein aggregation and neurodegeneration, *Curr Protein Pept Sci.* **12**, 258-268.
25. Pandey, A. P. & Sawant, K. K. (2016) Polyethylenimine: A versatile, multifunctional non-viral vector for nucleic acid delivery, *Mater Sci Eng C Mater Biol Appl.* **68**, 904-918.
26. Wang, X., Niu, D., Hu, C. & Li, P. (2015) Polyethyleneimine-Based Nanocarriers for Gene Delivery, *Curr Pharm Des.* **21**, 6140-6156.
27. Morris, V. B. & Labhasetwar, V. (2015) Arginine-rich polyplexes for gene delivery to neuronal cells, *Biomaterials.* **60**, 151-60.
28. Tung, C. H. & Weissleder, R. (2003) Arginine containing peptides as delivery vectors, *Adv Drug Deliv Rev.* **55**, 281-294.
29. Takeuchi, T. & Futaki, S. (2016) Current Understanding of Direct Translocation of Arginine-Rich Cell-Penetrating Peptides and Its Internalization Mechanisms, *Chem Pharm Bull (Tokyo).* **64**, 1431-1437.
30. Schmidt, N., Mishra, A., Lai, G. H. & Wong, G. C. (2010) Arginine-rich cell-penetrating peptides, *FEBS letters.* **584**, 1806-1813.

Figure Legends

Figure 1. Schematic of the approach used. Proposed tau peptides aggregation inhibition by cationic polymer polyethyleneimine and cationic polypeptide polyarginine.

Figure 2. Tau N peptide (100 uM) aggregation kinetics was studied with and without PEI and Arg at concentrations depicted in the figure. Thioflavin S fluorescence show significant inhibitory effect of both PEI **(A),** and Arg **(B)** on Tau N aggregation.

Figure 3. Tau V peptide (100 uM) aggregation kinetics was studied with and without PEI and Arg at concentrations as depicted in the figure. Thioflavin S fluorescence show significant inhibitory effect of both PEI **(A)**, and Arg **(B)** on Tau V aggregation.

Figure 4. Scrambled peptides aggregation kinetics was studied with Thioflavin S binding assay. Thioflavin S fluorescence, indicate the scrambled peptides did not exhibit significant peptide aggregation.

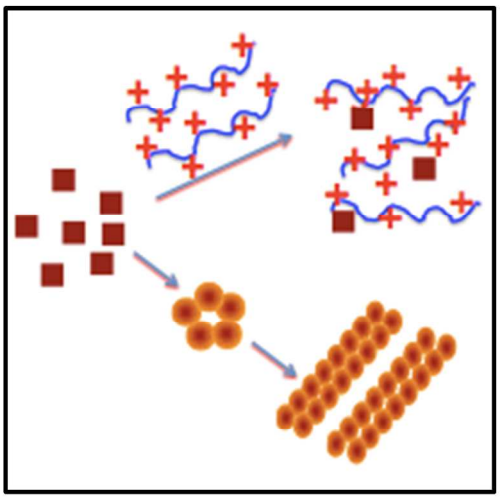
Figure 5. TEM images show the tau peptides aggregation inhibition in the presence of PEI and Arg. **(A)** Tau N, with and without PEI or Arg, **(B)** Tau V, with and without PEI or Arg. Scale bar 1 μm.

Figure 6. Dynamic light scattering measurements of the Tau N peptide aggregation with and without PEI **(A)** or Arg **(B)**, indicating the reduction in aggregation size in the presence of PEI or Arg.

Figure 7. Tau mutant protein (100 nM) aggregation inhibition studies show that PEI and Arg were able to inhibit protein aggregation **A)** Thioflavin S, **B)** Dynamic light scattering, and **C)** TEM images. Scale bar 1 µm.

Figure 8. XTT assay show no significant decrease on the cell viability of SH-SY5Y cells in the presence of **(A)** PEI or **(B)** Arg at few micromolar concentrations.

Figure 9. Tau N toxicity effect on SH-SY5Y cells. **A)** Tau N alone **B)** Tau N with 200 nM PEI **C)** Tau N with 2 µM Arg. * $p < 0.05$. Data are expressed as mean ± SEM.



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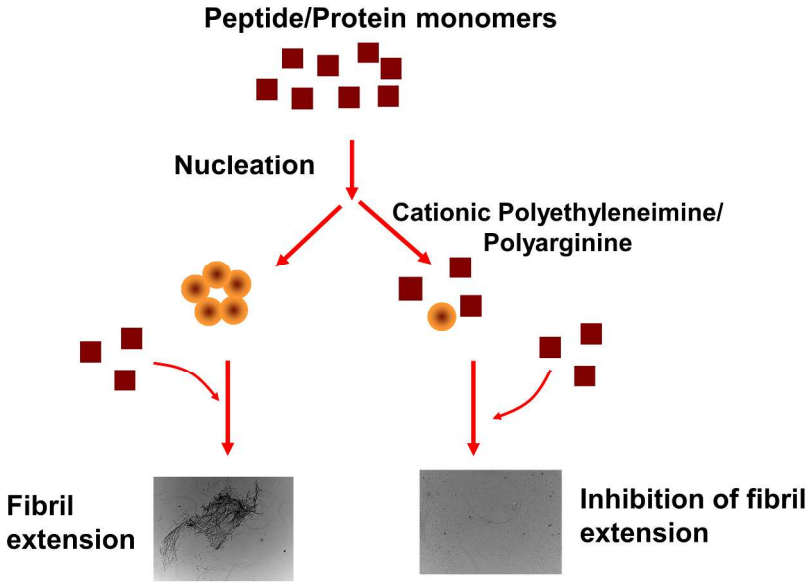

Figure 1

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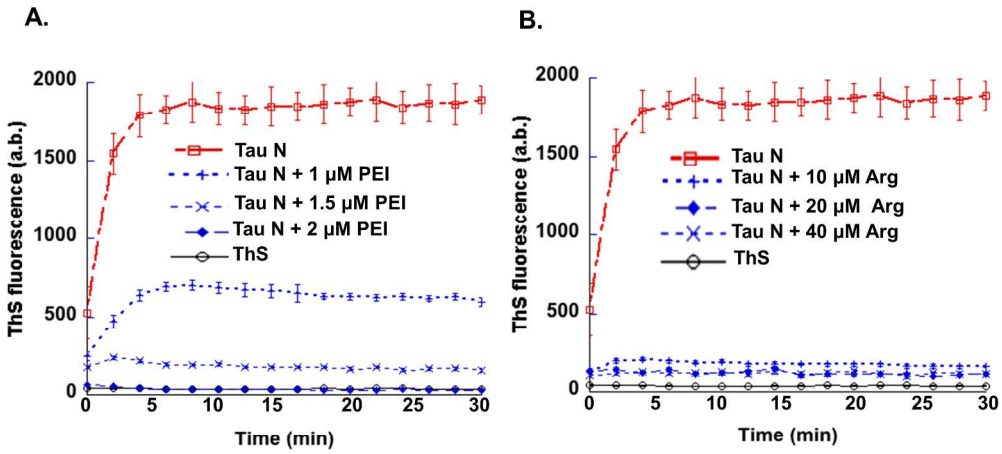

Figure 2

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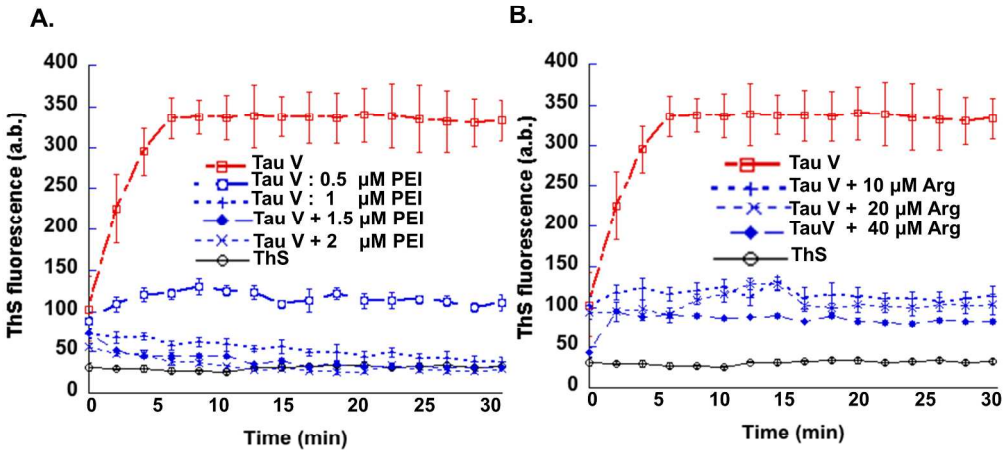

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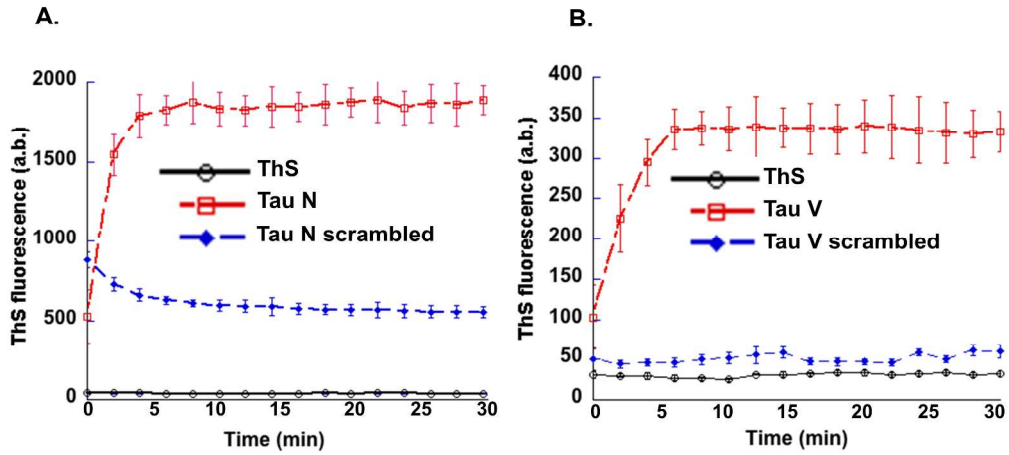

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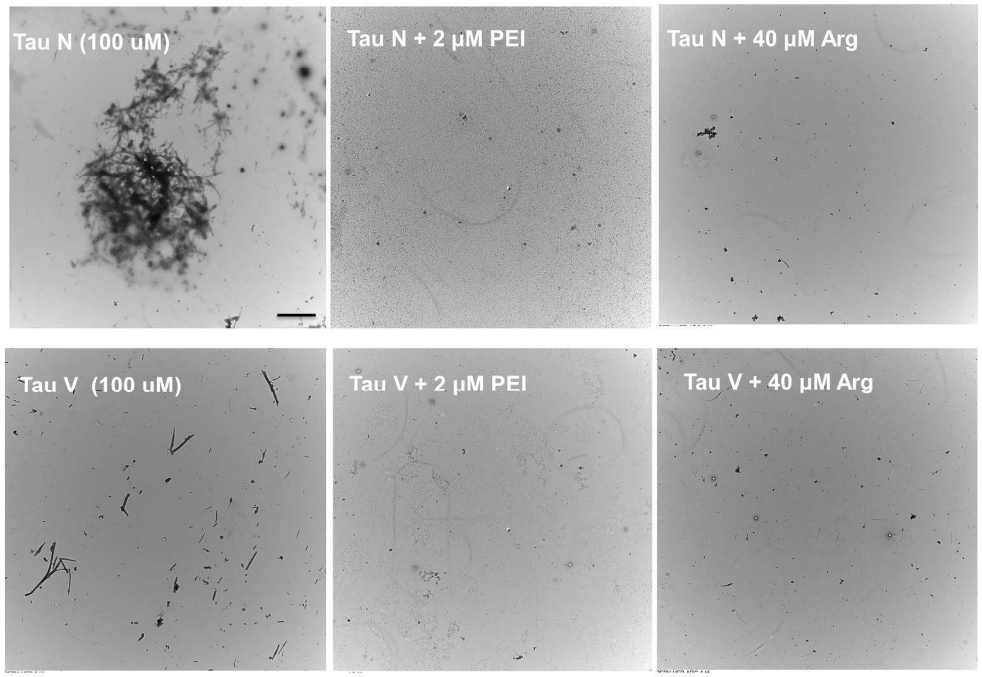

Figure 5

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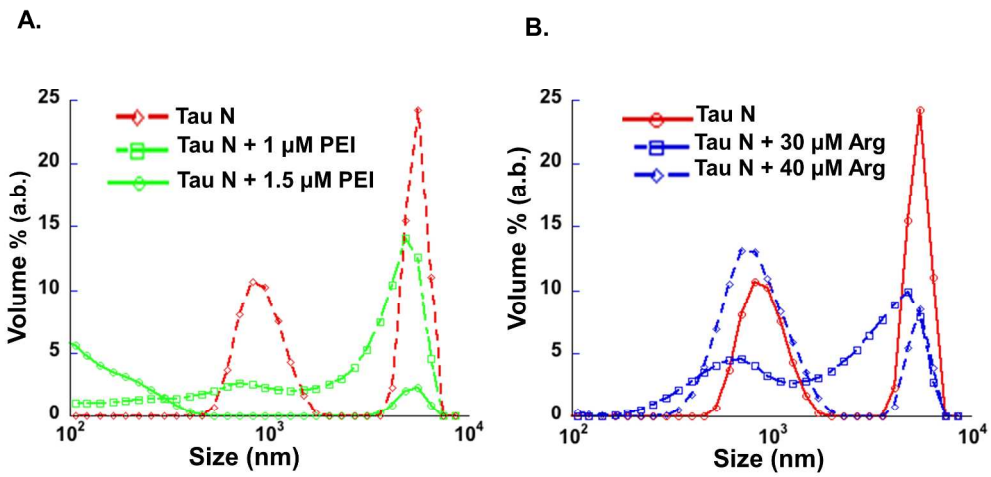

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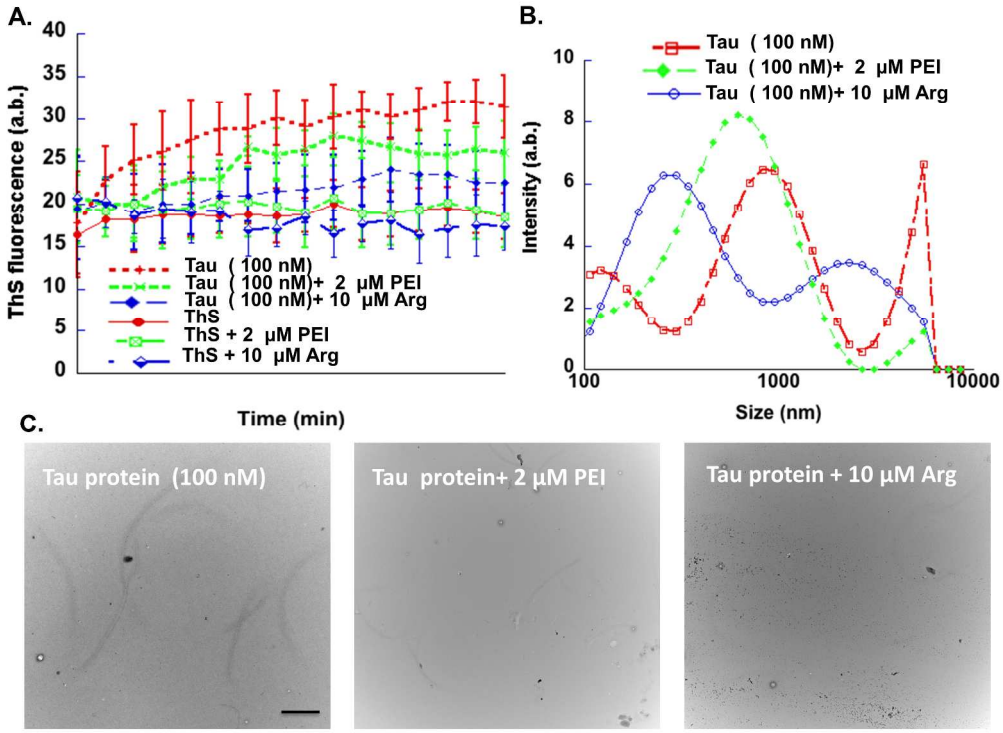

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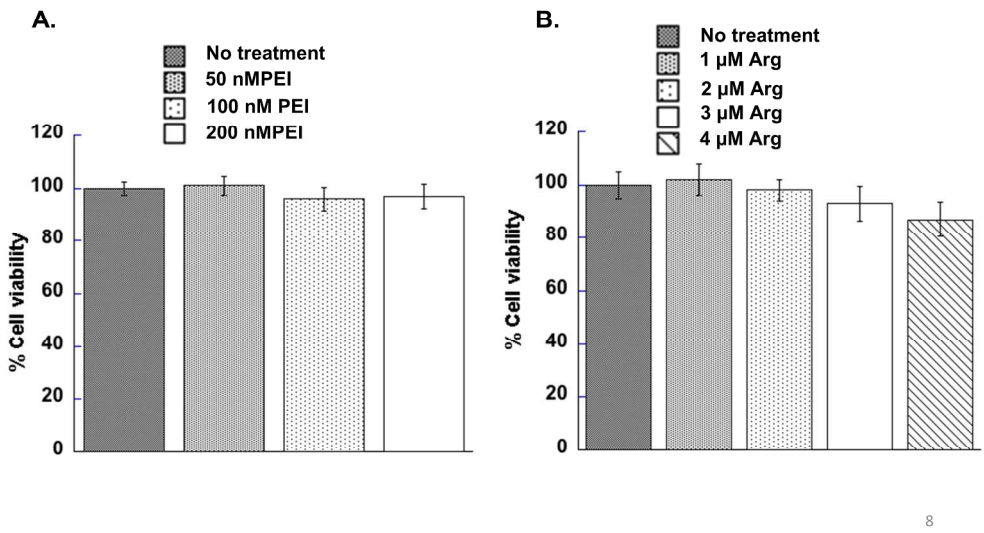

Figure 8

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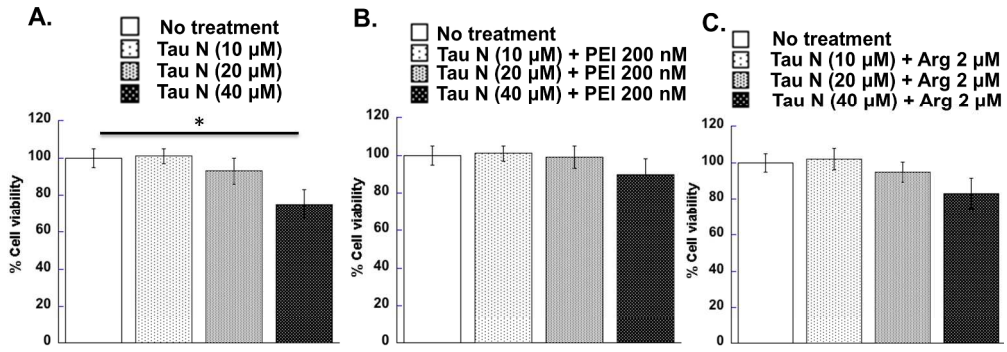

Figure 9

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